TOWARDS THE DEVELOPMENT OF AN IMPROVED VACCINE FOR SWINE AGAINST BRUCELLOSIS

A Thesis

by

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ABSTRACT

Swine brucellosis is a zoonotic disease with variable incidence rates in domestic and feral populations. It is a serious public health issue due to the potential for spillover not only between infected feral and domestic swine but also cattle and humans. Previous studies published by our laboratory has demonstrated the cross-protective immunity elicited by the S19Δ*vjbR* live attenuated vaccine in mice against challenge with *B. abortus, B. melitensis* or *B. suis,* suggesting its potential use as a vaccine against multiple *Brucella* species. To date there is no effective vaccine used to prevent brucellosis in swine. This prompted us to study the potential use of S19 Δ*vjbR* as a vaccine candidate in this natural host by evaluating its safety and humoral response in pregnant swine. Fifteen pregnant gilts at fifty days of gestation (midgestation) were divided into four groups and vaccinated subcutaneously with $1X10^{10}$ CFU of either; 1) strain S19; 2) S19Δ*vjbR* encapsulated in alginate microspheres 3) unencapsulated S19Δ*vjbR*, or 4) empty capsules as a control. Interestingly, none of the animals vaccinated with either S19 or S19Δ*vjbR* aborted or demonstrated any adverse side effect associated with vaccination. No bacteria was recovered from any of the tissues examined supporting the lack of histopathological changes in major organs either in piglets or in gilts. Vaginal swab culture from vaccinated animals showed no bacterial growth on Farrell's agar medium. RBT and IgG iELISA tests were found substantial at 2 week (k= 0.8) and 4 week (k=0.65) post-vaccination suggesting that RBT can be used during this interval as a good tool to confirm the immunization of animals with the different vaccine

strains. Different responses of gilts sera against purified *vjbR* protein at pre-vaccination and two week post-vaccination raised the question of specificity of the *vjbR* as marker and its inability to validate DIVA capabilities of the Δ*vjbR* vaccine candidates.

DEDICATION

This work is dedicated to the souls of my aunts Zohra and Wassila, my parents, Mohamed & Khadija and my sister Sonia for their encouragements throughout this journey. I hope that wherever you are you would be proud of me after this achievement.

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NOMENCLATURE

iELISA	Indirect Enzyme-linked Immunosorbent Assay
WB	Western blot
RBT	Rose Bengal Test
DIVA	Differentiating Infected from Vaccinated Animals
CFUs	Colony-forming units

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1. INTRODUCTION

Brucellosis is a zoonosis caused by *Brucella* spp of nearly worldwide distribution especially in low and middle income countries [1]. Among the 12 identified species of *Brucella* classified based on preferential host specificity, 3 are highly pathogenic not only for their preferred host species but also for humans (*Brucella melitensis* (sheep and goat), *Brucella abortus* (cattle) and *Brucella suis* (swine)), and are all associated with significant economic losses in different parts of the world [2]. The disease manifests differently in animals and humans. Brucellosis in humans is acquired by direct contact with animal discharges or consumption of unpasteurized milk products. It is considered a debilitating febrile disease with undulant fever as a major symptom, frequently accompanied with fatigue, sweats, malaise, chills, weight loss, arthralgia and myalgia [3]. Treatment is based on combined antibiotic regimen that can last for up to 6 months limiting the ability of patients to work or support their families [4,5]. Without adequate treatment, the disease can persist chronically. Several complications can be encountered including osteoarticular, cardiovascular, neurological and adverse obstetrical problems that can lead to abortion during the first or second trimester of pregnancy [6–9]. Brucellosis has been associated with occupational exposure in farmers, veterinarians and slaughterhouse workers. *Brucella* infection in animals is characterized by abortion and infertility in cattle, sheep, goats, swine and canines [10,11]. This leads to a serious problem in the trade of animals and their products causing significant economic losses for livestock and kennel breeders [12]. Countries that lack efficient control programs

based on quarantine, slaughter and vaccination are the most susceptible to these problems.

Swine brucellosis is encountered in domestic and feral pigs. The disease can be transmitted via venereal route with a high risk of spill-over between wild boar and outdoor domestic pigs as well as cattle [13]. It is associated with impaired infertility manifested by orchitis and epididymitis in boars, production of small litters, and abortion in sows that can occur at any stage of gestation [14]. However, if infection with *B. suis* occurs after day 35 of gestation, late abortion is usually observed [15]. There are five biovars of *Brucella suis* [16,17]. Only three of them, including biovar 1, 2 and 3, have been known to cause infection in swine [16,17]. *Brucella suis* biovar 1 and 3 are the main biovars that infect swine and have been reported in the United States and Australia as the main cause of human brucellosis especially in hunters of feral hogs [18–20]. *B. suis* biovar 2, whose natural carriers are hares, has been isolated from outbreaks in Europe from 1999 to 2000 from wild boar [21–23]. This is important due to the potential spillover from the feral swine to the domestic outdoor pig herds and the high zoonotic risk [21]. Other *Brucella* species, such as *B. abortus* and *B. microti,* may infect swine [24–26].

Swine brucellosis is the perfect example of the "One Health" paradigm in which vaccination represents a key strategy to protect animals and therefore humans [27,28]. Along these lines, enhancing veterinary vaccines can considerably reduce the burden of *Brucella suis* leading to improvement of public health [29].

Currently, there is no effective vaccine against swine brucellosis. Despite the availability of commercial *Brucella* live attenuated vaccines for cattle and small ruminants, their efficacy on domestic pigs is still controversial. *Brucella abortus* Strain 19, a spontaneously attenuated smooth *B. abortus* used since 1941 [30], is highly protective in cattle [31]. The major drawback is residual virulence to pregnant animals and interference with diagnostic tests due to the production of anti-LPS antibodies in immunized animals [2]. Studies of the potential use of S19 in swine have been very limited with mixed results in regards to their protective efficacy when animals were challenged with *Brucella suis* wild type [32–34]. Another vaccine that have been studied for its potential use in swine is the most recently developed *Brucella abortus* RB51 which is a live attenuated rough mutant of *B. abortus* strain 2308 that replaced S19 for the vaccination of cattle in the USA. Safety and efficacy studies in pigs using the RB51 demonstrated no protection against *B. suis* challenge in domestic pigs [35]. The only commercially available vaccine that is currently employed in swine is the strain 2 (S2) [36]. This strain was isolated from an aborted sow in 1953 and attenuated by serial passage [37]. Due to residual virulence associated with abortion when administered subcutaneously or intramuscularly, the vaccine is currently being administered only orally in China [36]. However, the major drawback is the lack of consistent levels of protection attributed to non-reproducible immunization doses via this route [38]. Studies against wild type *B. suis* challenge have demonstrated 75% protection in pregnant sows when vaccinated under controlled laboratory doses [36].

Recent advances in *Brucella abortus* vaccine development, outline the promising role of live attenuated vaccines via gene deletion or overexpression to enhance immune protection [2]. Various virulence factors have been identified, among them is the *VirB* regulon [39,40]. This virulence factor is essential for *Brucella* to replicate inside macrophages and sustain infection [41]. Research in our laboratory has identified *Brucella* spp genes required for virulence and survival via transposon mutagenesis [42,43]. Among these genes, *vjbR* (BMEII1116), encodes a LuxR-like transcriptional regulator involved in quorum-sensing, and required for adequate *VirB* (T4ss) expression [44]. *VjbR* mutants have been proven to be highly effective in inducing protective efficacy in different animal species when exposed to wild type *Brucella*. Our laboratories have demonstrated that *vjbR* mutants can induce cross-protective immunity, suggesting its potential use in different animal species against different virulent *Brucella* spp [45,46]. This study evaluates the potential use of live attenuated *Brucella abortus* strain 19 Δ*vjbR* as a vaccine to be used in swine regardless of their reproductive status. S19 Δ*vjbR* mutants have been demonstrated to be safer than S19 when inoculated in different laboratory animals [47,48], however, the protective efficacy is diminished compared to S19. To overcome the reduced efficacy, we have previously demonstrated that efficacy can be enhanced by delivering the vaccine in a microencapsulated format. The vaccine strain is engulfed within an erodible alginate microcapsule containing non-immunogenic protein designated VpB (Vitelline protein B), that is extracted from the eggshell of *Fasciola hepatica* [49]. The decay of the capsule gradually releases the Δ*vjbR* knockout mutant over a period of 36 days [50].

Based on the promising findings of vaccinating animals with live attenuated S19Δ*vjbR* vaccine strains, we sought in this study to evaluate the safety of S19 Δ*vjbR* vaccine candidates in comparison to S19 in pregnant swine at mid-gestation, period of which they are more susceptible to abortion [15]. To monitor for vaccination, a follow-up of the humoral response was assessed biweekly by Rose Bengal Test (RBT), anti-*Brucella* IgM and IgG iELISAs until delivery. Further, purified *vjbR* protein was selected as a serological marker considering its immunodominant characteristic [51] and its ability identify *Brucella* infected humans using serodiagnostic tests [52]. These factors prompted the use of purified *vjbR* protein to differentiate vaccinated gilts with Δ*vjbR* vaccine candidates from those vaccinated with S19 and consequently establish a serological test that demonstrates the DIVA capability of the vaccine candidates.

2. MATERIALS AND METHODS

2.1. Animals

Fifteen American Yorkshire healthy gilts were obtained from a privately owned swine herd in Texas. All gilts were synchronized and artificially inseminated at the same time. Upon arrival, all animals were confirmed to be negative for brucellosis. At 50 day post-insemination, pregnancy was confirmed via ultrasound. Only pregnant animals were included in the study. The animals were housed in an outdoor fenced Biosafety Level 2 area with restricted access at the Veterinary Medical Park of Texas A&M College of Veterinary Medicine and Biomedical Sciences and acclimated for 1 week prior to vaccination. The experimental protocol was approved by TAMU Institutional Animal Care and Use Committee (IACUC).

2.2. Construction of the S19 Δ*vjbR* vaccine candidate

S19 Δ*vjbR* deletion was engineered and used as a vaccine candidate in a previous study [48]. Specifically, for this mutant, the sequence downstream of the *vjbR* gene was amplified from *B. abortus* 2308 with the primer pair 5′-GTCTTCGAGGATGTACAATTGGC and 5′-CATCTCGTCTGATCAACATGG. The sequence upstream of *vjbR* was amplified with the primer pair 5′-GAAGCGCCAAAGTATCGC and 5′-CAGTTGGAAAAGGGCTTTTCCAACCG.

These two products were ligated to one another via overlapping PCR with an AscI site (New England Biolabs) engineered between the two sequences.

This product was then ligated to pEX18Ap, and a kanamycin resistance gene was inserted within the vector at the unique AscI site. This construct was used for the electroporation into S19. Potential marked deletion mutants were kanamycin resistant and ampicillin sensitive and were verified by PCR and Southern blotting.

2.3. Preparation of *Brucella abortus* S19 and S19 Δ*vjbR* vaccine strains

Brucella abortus S19 (NVSL, Ames, IA) and S19 Δ*vjbR* were grown separately on Tryptic Soy Agar plates (TSA) for 3 days at 37°C with 5% (vol/vol) CO_2. The vaccine strains were harvested from the surface of the plates after 3 days of incubation using phosphate-buffered saline (PBS), pH 7.2. The bacteria were resuspended to a final concentration of 1×10^{10} CFU/mL based on optical density readings using a Klett meter and a standardized light-scattering curve. Actual viable counts were confirmed retrospectively by serial dilution, plating, and enumeration.

2.4. Preparation of encapsulated *B. abortus* S19 Δ*vjbR* vaccine strain

Vaccine preparation was performed as previously described [48]. Briefly, 1×10^{10} CFU of the *B. abortus* S19 Δ*vjbR* was resuspended in 1 ml of MOPS buffer (10 mM MOPS, 0.85% NaCl [pH 7.4]) and mixed with 5 ml of alginate solution (1.5% sodium alginate, 10 mM MOPS, 0.85% NaCl [pH 7.3]). Extrusion of the suspension through a

200-μm nozzle into a 100 mM calcium chloride solution produced spheres that were stirred for 15 min by using a Nisco Encapsulator Var V1 (Nisco Engineering AG, Zurich, Switzerland). The capsules were then washed twice with MOPS for 5 min and further cross-linked with 0.05% poly-l-lysine (molecular weight. 22,000; Sigma) for 10 min. 2.5 mg of VpB (vitelline protein B [45]) was added to the crosslinking solution. After two successive washes, the microspheres were stirred in a solution of 0.03% (wt/vol) alginate for 5 min to apply a final outer shell and washed twice with MOPS before storage at 4°C.

2.5. Cloning, expression and purification of the *vjbR* recombinant protein

The *vjbR* BMEII1116 open reading frame (ORF) was amplified by PCR from *Brucella melitensis* 16M then cloned into Champion pET SUMO N-terminal 6xhist-tagged vector expressed in *E. coli* BL21. Glycerol stock of *E. coli* carrying *vjbR* protein was cultured in 10ml of LB broth overnight at 37°C. Plasmid DNA was isolated according to the manufacturer instructions (E.Z.M.A Plasmid DNA Mini kit (Omega bio-tek)) and quantified using Nanodrop spectrophotometer. Sequencing of the plasmid DNA (~400 ng/ sample) was done using pET Sumo vector forward sequencing primer and using *vjbR* 3' end reverse primer separately. The expression of the recombinant protein was assessed by performing an SDS-PAGE to the bacterial lysate containing the protein. The *vjbR* protein was purified from the BL21 lysate expressing the fusion protein by nickel column affinity chromatography using the ProBond™ Purification System Kit (Invitrogen) and analyzed by SDS-PAGE. The protein was dialyzed against

10 mM Tris, pH 8.0, 0.1% Triton X-100 overnight at 4°C to remove the urea and protein concentration was measured using Pierce BCA protein assay kit (Thermofisher).

VjbR protein was also expressed in yeast and antibody against *vjbR* was raised in rabbit (MyBiosource, San Diego, USA) to use it for the characterization of the purified protein.

2.6. Characterization of the *vjbR* protein via liquid chromatography–mass spectrometry

Polyacrylamide gel containing *vjbR* protein stained with traditional Coomassie stain was transferred to Protein Chemistry Laboratory of Texas A&M University. An individual specific band of ~40 kDa was excised then reduced from disulfide bonds and alkylated with iodoacetamide prior to digestion. After tryptic digestion, the sample was analyzed on a ThermoFisher LTQ linear ion trap mass spectrometer using nano-LC peptide separations [53].

2. 7. Determination of *vjbR* like protein homology in other bacteria and assessment of crossreactivity

The *vjbR* protein sequence was subjected to protein-protein BLAST (Basic Local Alignment Search Tool; blastp) analysis from the NCBI website to identify sequence similarity of the *vjbR* protein with other organisms via sequence alignments. *Ochrobactrum anthorpi*, *Pseudomonas aeruginosa* and *E. coli* strain K-12 were selected for their close similarities with the *vjbR* protein to assess for crossreactivity. Colonies of

Ochrobactrum anthorpi were grown on TSA plates and *Pseudomonas aeruginosa* and *E. coli* strain K-12 were grown on Luria Bertani (LB) agar plate at 37°C for 48 hours. 5 mL of PBS was added to each plate and mixed with the bacteria and then collected into 10 mL tubes. The suspension was centrifuged for 5 minutes at 5000 rpm then the supernatant was discarded. Guanidine lysis buffer was added to the pellet for 10 minutes shaking then subjected to sonication. Then, the solution was centrifuged for 10 minutes at 13000 rpm. Supernatants were collected and subjected to SDS-PAGE followed by transfer into nitrocellulose membrane for western blot analysis. Pre-vaccinated gilt sera from S19 and S19 Δ*vjbR* encapsulated were used as primary antibodies (1:5000) for the detection of any cross-reactivity with *Ochrobactrum anthorpi*, *Pseudomonas aeruginosa* and *E. coli* strain K-12 lysates.

2.8. Immunization of pregnant gilts

Confirmed pregnant gilts by ultrasound at 50 days of gestation were randomly distributed into 4 groups and inoculated subcutaneously (SQ) in the scapular area with a single dose containing $1x10^{10}$ CFU of either 1) S19 (n=4), 2) encapsulated S19Δ*vjbR* (n=4), 3) unencapsulated S19Δ*vjbR* (n=4), or 4) control (n=3). All animals were housed individually and no direct contact between animals of different groups was allowed.

2. 9. Clinical evaluation

2. 9. 1. Monitoring for adverse side effects associated with vaccination

Prior to vaccination and until delivery, gilts were monitored twice a day for any adverse side effects associated with vaccination including abortion, adverse reactions at the site of injection, abnormal vaginal discharges, and fever. Rectal temperature of pregnant gilts was measured daily using a digital thermometer to assess any abnormal temperature fluctuation. 38.8ºC (± 0.3°C) was considered the normal body temperature threshold for gilts [54].

2. 9. 2. Vaginal shedding of the vaccine strains

Screening for vaginal shedding of the different vaccine strains was performed biweekly on all animals. Vaginal swab specimens were obtained individually from all gilts and plated onto Farrell's agar medium (OXOID) for bacterial isolation. Plates were incubated at 37°C and monitored daily for up to 30 days.

2. 9. 3. Gross and histopathological evaluation of gilts

Within the first 3 days post-delivery, all sows were euthanized via pentobarbital overdose and necropsied for the detection of any gross lesions associated with vaccination. Multiple tissue sections including: spleen, liver, lung, uterus, placenta, pre-scapular, mammary, inguinal and mesenteric lymph nodes were collected from sows and transferred into histology cassettes. Tissue sections were fixed in 10% buffered formalin, paraffin embedded and section stained with hematoxylin and eosin (H&E). Histological

changes between different treatment groups were microscopically assessed by a board certified veterinary anatomic pathologist.

2. 9. 4. Determination of bacterial colonization in tissues from gilts

Colonization of maternal tissues was assessed by culture after delivery. Spleen, liver, lung, uterus, pre-scapular, mammary, inguinal and mesenteric lymph nodes were removed and weighed. 1gr of tissue was homogenized and resuspended in 1 mL of PBS using Omni Prep Multi-Sample Homogenizer (Omni-inc, GA, USA). Serial dilutions were prepared and 100 μL of each sample was plated onto TSA (Tryptic Soy Agar) and Farrell's agar medium (OXOID), and incubated up to 30 days at 37°C with 5% (vol/vol) CO_2. The experimental limit of detection was determined to be 10 CFU per gram of tissue.

2. 9. 5. Gross and histopathological evaluation of piglets

Piglets from all gilts were euthanized within the first hours of birth. All the piglets were necropsied and evaluated for gross lesions. Lung float test was conducted to assess fetal viability upon birth. Tissue sections of spleen, liver, lung and umbilical cord were fixed in 10% buffered formalin, paraffin embedded and section stained with hematoxylin and eosin (H&E). Histological changes between different treatment groups were microscopically assessed by a board certified veterinary anatomic pathologist.

2. 9. 6. Vertical colonization of piglet tissues

In an effort to determine if the vaccine strains were capable of colonizing piglet tissues during gestation, sections of spleen, lung, liver, kidney, pylorus and umbilicus of all piglets were collected within the first 12 hours of delivery. 1 gr of tissue from each organ was cultured in TSA (Tryptic Soy Agar) and Farrell's agar medium (OXOID) after homogenization in 1 mL of PBS using Omni Prep Multi-Sample Homogenizer (Omni-inc, GA, USA) and incubated up to 30 days at 37°C with 5% (vol/vol) CO_2. The experimental limit of detection was determined to be 10 CFU per gram of tissue. All cultures were done in duplicate.

2. 10. Serological responses in pregnant gilts

2. 10. 1. Rose Bengal Test (Brucellosis Card Test)

Approximately 10 mL of blood was collected from each gilt into tubes without anticoagulant prior to vaccination and at 2, 4 and 6 week post-vaccination. Blood was centrifuged at 2000 x g for 15 min to collect serum and stored at -20°C. Serum agglutination against *Brucella* antigen was performed using the brucellosis Card Test (Becton Dickinson Microbiology Systems for USDA/APHIS National Veterinary Services Laboratory, Ames, Iowa). 30 µL of sera from immunized animals was added to an equal amount of Rose Bengal antigen into a brucellosis diagnostic card and mixed thoroughly with a stick. A scale was developed to categorize the degree of agglutination and consisted of: 1) ++++/+++ strong agglutination, 2) ++ mild agglutination, 3) + weak agglutination and 4) - no agglutination

2. 10. 2. Determination of anti-*Brucella* IgMs and IgGs

Indirect Enzyme Linked Immunosorbent Assay (iELISA) to detect anti-*Brucella* specific immunoglobulin G (IgG) and M (IgM) was performed prior to vaccination and at 2, 4 and 6 weeks post-vaccination serum samples in polystyrene microtiter plates (Maxisorp, NUNC). 96 well plates were pre-coated overnight at 4°C with 25 µg/well of *Brucella abortus* 2308 heat killed/sonicated lysate as antigen. The next day, plates were washed with PBS-T to remove unbound proteins, blocked with 0.5 ml of blocking buffer (0.25% [wt/vol] bovine serum albumin), and then incubated with sow sera samples (dilution 1:500) in the same blocking buffer for 1 h at room temperature. After extensive washing to remove unbound antibody, the secondary antibody (peroxidase labeled goat anti-swine IgG or IgM) was added at a dilution of 1:1000, and incubated for one hour. At the end of the incubation period, plates were washed again, and horseradish peroxidase substrate was added, followed by incubation for 15 min. The reaction was stopped by adding 50 µl of 0.5 M NaOH, and the absorbance was measured at 450 nm (*A*450). All assays were performed in triplicate. The results are represented as the mean of triplicate wells for each sample.

2.11. Immunodetection of the *vjbR* his-tagged recombinant protein expressed in *E. coli* and in yeast

The expression of *vjbR* recombinant protein was examined by western blot. An SDS-PAGE with the purified *vjbR* expressed *in E. coli*, the *E. coli* lysate containing the *vjbR* gene in the plasmid as a positive control and *E. coli* without plasmid as a negative control, were transferred into a nitrocellulose membrane for 120 min (100 V). Another

SDS-PAGE using the histagged *vjbR* expressed in yeast was run separately (*results not shown*). After incubation with 5% nonfat dry milk for 2 hours, the membranes were washed 3 times with TTBS 0.2% (5 min each wash) then incubated with mouse HRP anti-histag antibody (1:20000) shaking in the dark at +4 ^{o}C overnight.

The following day, the nitrocellulose membrane was washed 6 times with TTBS 0.2% and then incubated with Clarity™ Western ECL Blotting substrate for 5 min).

2. 12. Polyclonal anti-*vjbR* antibody production

One rabbit was immunized with the purified *vjbR* expressed in yeast for anti-*vjbR* antibody production. After three boosters of the *vjbR* protein, the serum was collected and used as primary antibody (1:5000 dilution) after transfer of the *vjbR* protein into a nitrocellulose membrane. Peroxidase labeled anti-rabbit total IgG (1:10000) was used as secondary antibody. Clarity™ Western ECL Blotting substrate (Bio-Rad, CA, USA) was used for detection. Images were analyzed using EMBL ImageJ software. A band size of ~40 KD expressing the *vjbR* was observed.

2. 13. Evaluation of the potential use of the *vjbR* protein as a DIVA marker

The ability of differentiating vaccinated animals with S19 Δ*vjbR* from those vaccinated with S19 was assessed by a western blot based assay using the *vjbR* as antigenic marker.

2. 13. 1. Western blot based assay

The *vjbR* protein expressed in *E. coli* was subjected to 12% SDS-PAGE followed by transfer into nitrocellulose (NC) membrane using wet electroblotting system (Bio-Rad, CA, USA). Sera from all pre-vaccinated and 2 week post-vaccination gilts were used as primary antibodies. Peroxidase labeled goat anti-swine total IgG (KPL, MA, USA) was used as secondary antibody. Multiple serum titrations of primary and secondary antibodies ranging from 1:2500 to 1:20000 were tested and finally 1:5000 dilution for primary antibodies and 1:10000 for secondary antibody were selected for downstream experiments. Positive control was included and consisted of the rabbit anti-*vjbR* primary antibody (MyBiosource, CA, USA) (1:5000) and Peroxidase labeled anti-rabbit total IgG (KPL, MA, USA) was used as secondary antibody (1:10000). Clarity™ Western ECL Blotting substrate was used for detection. Presence of *vjbR* band (~40 KD) in S19 vaccinated animals and absence of *vjbR* band in S19 Δ*vjbR* was expected.

2.14. Statistical analysis

All analyses were performed using the GraphPad Prism 6.0 software (San Diego, CA, USA) and *P* values <0.01 were considered significant. The non-parametric one-way analysis of variance (ANOVA) test was used to compare between litter size temperature of different groups and Tukey's multiple comparisons was used to generate *P* value. The non-parametric one-way analysis of variance (ANOVA) test was used to compare between body temperature of different groups and Dunn's multiple comparisons was used to generate *P* values for selected mean comparisons. The cut-off value of the

iELISA (0.4) was calculated as three standard deviations plus the average of negative controls. The two-way analysis of variance (ANOVA) test was used for the anti-*Brucella* IgG and IgM experiments followed by Tukey's multiple comparisons test. Correlation between the iELISA total IgG and the Rose Bengal Test results was evaluated by Pearson's correlation coefficient. Kappa (κ) statistic was used to establish the degree of concordance between the two assays.

3. RESULTS

3. 1. Clinical evaluation in gilts

3. 1. 1. Monitoring of abortion associated with the use of S19 Δ*vjbR* and S19 vaccine candidates

One of the main drawbacks associated with vaccination using LAV is the induction of abortion in pregnant animals [55–59] . In order to determine if vaccination with either S19 or S19 Δ*vjbR* induced abortion, animals were vaccinated subcutaneously (SQ) at a dose of $1X10^{10}$ CFU with the different vaccine candidates at mid-gestation and were monitored daily until farrowing. Interestingly, all the animals farrowed normally regardless of the treatment group with no evidence of abortions or preterm delivery observed (Table 1).

The average litter size per group was 12 animals in both vaccinated S19 and S19 Δ*vjbR* encapsulated groups, 11 animals in S19 Δ*vjbR* unencapsulated group and 12 animals in the control group. There was no significant difference in litter size among different groups (*p-value* 0.9) suggesting that vaccination did not influence litter size.

Delivery in all gilts was characterized by the normal delivery of healthy piglets among all groups. Post-partum period was characterized by behavioral changes in gilts manifested by rejection of newborns and deaths by crushing among different groups.

In order to distinguish between traumatic deaths and stillbirths, lungs of all dead piglets were submitted to lung test by embedding a part of lung from each dead piglet into water [60]. 16.66% of piglets from S19 and control groups were confirmed as

stillbirths (Table 1). Interestingly, no stillborns were detected among S19 Δ*vjbR* encapsulated and unencapsulated groups. So far, the significance of this finding does not relate stillbirths to vaccination since the highest mortality including stillborn piglets was observed among the control group.

3. 1. 2. Clinical examination and body temperature determination

Gilts were monitored daily to evaluate any adverse side effects associated with vaccination. Throughout the study period, no adverse effect associated with behavior, loss of body weight, or local inflammation response at the injection site were observed among the different groups (*data not shown*).

Assessment of body temperature was performed daily via rectal route. Fever was considered to be a temperature above 38.8°C [54]. No significant changes in body temperature were observed between groups regardless of the vaccine formulation (Fig.1). Although not significant, decrease in body temperature in all groups was observed between 2 and 3 week post-vaccination which corresponded with low environmental temperatures of 9 to 8ºC (Fig. 1).

3.1. 3. Vaccine shedding in vaginal secretions

A major drawback of the use of LAV is the possibility of the vaccine to be excreted into the environment, serving as potential source of contamination and infection of naïve animals or non-target species [61].

In an attempt to determine if any of the vaccine formulations were shed into the environment through vaginal secretions, vaginal swabs cultures were done biweekly starting from the day of vaccination until delivery. There was no growth on Farrell's agar medium in any of the animals regardless of the vaccine formulation (Table 2).

3. 1. 4. Determination of bacterial colonization in gilts

The kinetics of bacterial clearance, at the time of delivery was evaluated by tissue culture. Liver, lung, spleen, lymph nodes, uterus and placenta were collected after euthanasia of all gilts within 5 days of the delivery. Interestingly, no bacteria was recovered from any of the tissues examined after delivery (Table 3).

3. 1. 5. Gross and histopathological evaluation of major tissues in vaccinated gilts

Previous studies in the mouse model using *Brucella abortus* S19 LAV have demonstrated that major organs can display inflammatory responses consequent to vaccination [62].

In mice, vaccination with S19 is characterized by the induction of splenomegaly, considered to be a classic sign of brucellosis. Mice inoculated with S19 Δ*vjbR* do not cause this inflammatory response [47,48]. Based on these observations, determination of any histopathological changes secondary to vaccination was assessed in major organs of vaccinated gilts. A full necropsy was conducted in all gilts within the first five days post-delivery. Tissue sections consisting of liver, lung, spleen and uterus were collected,

formalin fixed, paraffin embedded and double blinded and analyzed by a board certified anatomic pathologist.

No significant histopathological changes were observed in all major organs (spleen, liver, lung, uterus) of gilts regardless of the treatment (Fig. 2).

3. 2. Serological responses in pregnant gilts

3. 2. 1. Rose Bengal Test (Brucellosis Card Test)

The humoral response elicited by the vaccination of gilts was evaluated biweekly over the course of 6 weeks. The RBT was used as a rapid screening test for vaccination.

A summary of the results is presented in table 4. Serum samples from control animals and at pre-vaccination and control were negative. At 2 week post-vaccination, the agglutination response was the highest among all groups regardless of the formulation. There was no statistical difference between the different vaccine candidates. Starting at 4 week post-vaccination, the agglutination response started to decrease in all groups and varied between mild or weak agglutination, with only 2 animals from the S19 Δ*vjbR* unencapsulated still remained as strong positives. At 6 week post-vaccination, regardless of the treatment, all gilts became serologically negative. One gilt vaccinated with unencapsulated S19 Δ*vjbR* remained negative during the whole period of the experiment and later confirmed to have seroconverted via ELISA.

3. 2. 2. Determination of anti-*Brucella* IgM and IgG

To further elucidate the kinetics of the humoral response elicited by the inoculation of different vaccine strains, indirect ELISAs were conducted to measure the anti-*Brucella* IgM and IgG OD values using *B. abortus* 2308 heat killed lysate as the coating antigen. Anti-*Brucella* IgM and IgG OD values were negative in all groups prior to vaccination. At 2 week post inoculation, the anti-*Brucella* IgM in all vaccinated animals was the highest throughout the course of the study. Starting from 4 week post vaccination, the levels started to decrease in all animals and significant difference was noticed between S19 and S19 Δ*vjbR* encapsulated, S19 Δ*vjbR* unencapsulated and S19 Δ*vjbR* encapsulated but also with the control group ($P<0.01$). By 6 week post-vaccination there was no significant difference among all treatment groups and the control (Fig 3, upper panel). The kinetics of the anti-*Brucella* IgG levels was similar to that observed in IgM. The highest OD value was observed at 2 week post-vaccination among all vaccinated groups and no statistical difference was observed between vaccinated groups while the control group remained negative and statistically significant in comparison to them ($P<0.01$). At 4 week post inoculation, the levels of IgG in vaccinated animals had already started to decrease and remained statistically similar between each other but were still statistically significant compared to the unvaccinated group (Fig. 3. Lower panel). At 6 week post-vaccination, all gilts were negative (P value compared to control). As expected, the seronegative animal from S19 Δ*vjbR* unencapsulated group via RBT was confirmed to be positive by iELISA. The kinetics of both anti-*Brucella* IgM and IgG demonstrated a transient, short humoral response in

swine after vaccination regardless of the formulation which is different from what it is typically observed in mice, cattle or red deer inoculated with S19 or S19Δ*vjbR*.

The variation of the results obtained between the RBT and IgG iELISA at different time points prompted us to evaluate the correlation between the two techniques. The cutoff value for the indirect ELISA was calculated as three standard deviations plus the average of negative controls and it was equal to 0.4. The kappa value and the likelihood estimates were calculated using a Bayesian model [64].

Seroconversion of the vaccinated gilts at 2 week post-vaccination was at its highest by agreement of the iELISA and RBT (k= 0.8). The agreement between RBT and IgG iELISA tests was found substantial at 2 week (k= 0.8) and 4 week (k=0.65) post-vaccination (Table 5) suggesting that RBT can be used during this interval as a good tool to confirm the immunization of animals with the different vaccine strains.

During 6 week post-vaccination time point, the agreement between the two techniques was poor. This can be related to individual vaccinated animals that demonstrated higher OD values than the threshold (0.4) during that period (Fig. 3).

3. 3. Clinical evaluation in piglets

3. 3. 1. Vertical colonization of piglets

Previous studies have demonstrated that *Brucella* LAV candidates can cross the placenta and colonize fetal tissues resulting in the dissemination of the vaccine strains into the environment after delivery or abortion [65]. Vertical transmission of the vaccine strains to the offspring and colonization of different tissues of piglets was assessed by

culture of homogenized major tissues. Liver, spleen, lung, umbilicus, kidney and gastric contents were cultured. The lung and the gastric contents were included in this study, since these are two of the most common tissues to be colonized by *Brucella* in fetuses [18].

Regardless of the formulation or vaccine strain, there was no evidence of tissue colonization (limit of detection is 10 CFU/gr) in the samples analyzed. (Table 6).

3. 3. 2. Gross and histopathological evaluation of tissues from piglets

A complete gross and histopathological evaluation in all piglets was conducted in all piglets. A total of 181 piglets were subjected to analysis. Samples consisted of liver, lung, spleen, umbilicus, kidney and stomach. Interestingly, there were no significant changes in any of the groups and major organs commonly affected in the case of brucellosis in newborns such as umbilicus, lung and liver were unremarkable (Fig. 4).

Fetal deaths observed following farrowing and suspected to be associated with traumatic events were confirmed on gross and histopathological examination. In such cases, the presence of acute hemorrhage was consistently observed.

In the cases of stillbirths (total of 3), there was no evidence of an inflammatory or infectious process ongoing in these fetuses and were considered to be unrelated to the vaccination. Two of the stillborns observed were in the control, non-vaccinated group.

3. 4. Expression, purification and characterization of the *vjbR* fusion protein

The expression of the histagged *vjbR* protein was obtained after the induction of *E. coli* containing the Champion ™ pET SUMO plasmid (ThermoFisher scientific, NY, USA) with 1 mM IPTG. Following cell lysis, the *vjbR* fusion protein was purified according to the manufacturer instructions (ThermoFischer, NY, USA) and dialyzed against 10 mM Tris, pH 8.0, 0.1% Triton X-100 overnight at 4°C to remove the urea from the eluted buffer.

The concentration of the purified *vjbR* recombinant protein was measured via Pierce BCA protein assay (USA). The purified *vjbR* protein expressed in *E. coli* was subjected to 12% SDS-PAGE and *vjbR* band of around 40 kDa was excised for liquid chromatography-mass spectrometry (LCMS) to confirm the identity of the protein.

Scaffold Viewer software was used to compare mass spectrometry features to the database of the predicted protein (HTH-type quorum sensing-dependent transcriptional regulator *vjbR*). The results demonstrated a peptide coverage of 38% and *vjbR* was identified by 19 unique spectra and 9 unique peptides.

3. 5. Immunodetection of the *vjbR* protein

Anti-His-tag monocolonal antibodies were used for detection of histagged recombinant *vjbR* protein expressed in *E. coli* in comparison with appropriate controls. Positive control constituted by crude *E. coli* containing the plasmid expressing the *vjbR* and a lyste of *E. coli* without any plasmid serving as negative control were used (Fig. 5 A). Anti-histag monoclonal antibody detected the presence of the histidine tag *vjbR*

fusion protein band (～40 kDa) in both purified protein fraction as well as crude lysate of *E. coli* expressing *vjbR* but not in *E. coli* lysate lacking the *vjbR* expression (Fig. 5 A).

Similarly, the purified *vjbR* protein expressed in *E. coli* exposed to the rabbit anti-*vjbR* polyclonal antibody reacted to the same band size ～40 kDa (Fig. 5 B). Hence, we successfully characterized the *vjbR* protein with these two methods and these findings are compatible with the ones of the LC-MS.

3. 6. Evaluation of the potential use of the *vjbR* protein as a DIVA marker

3. 6. 1. Reactivity of gilt sera using western blot

Sera from all *Brucella*-free gilts at pre-vaccination (RBT and iELISA results) and sera from vaccinated groups and control group at 2 week post-vaccination were tested in Western blotting for reactivity with recombinant *vjbR* expressed in *E. coli*. Pre-vaccinated *Brucella*-free gilt sera from different groups elicited faint to strong unexpected immunoreactivity to the *vjbR* protein expressed in *E. coli.* Similar results to gilt sera at pre-vaccination were observed at 2 week post-vaccination by reacting non-specifically with the *vjbR* recombinant protein band (Fig. 6). Several titrations of gilt sera as well as anti-swine total IgG were used, however the non-specific binding of sera with the *vjbR* protein remained present.

3. 6. 2. Immunodetection of *vjbR* like protein reactivity with pre-vaccinated gilt sera

In an effort to understand the non-specific reactivity of gilt sera to the *vjbR* protein, analogy of the *vjbR* was investigated in different bacteria using NCBI sequence BLAST. BLASTp of the *vjbR* protein sequence revealed 79% homology with *Ochrobactrum anthropi*, 29% with *Pseudomonas aeruginosa* and 28% homology *with E. coli* K-12. Sera from animals that demonstrated a strong cross-reactivity with *vjbR* from different pre-vaccinated groups (S19 and S19 Δ*vjbR* encapsulated) were subjected to western blot analysis using lysates from these bacteria. Despite sharing variable homology with the *vjbR*, none of the animals demonstrated reactivity to the bacterial lysates of the correspondent organisms. Additionally, the rabbit anti-*vjbR* antibody did not react with any of the lysates (Fig. 7), ruling out the fact that none of that have the entire *vjbR* sequence.

4. SUMMARY AND CONCLUSIONS

Swine brucellosis is a zoonotic infectious disease caused naturally by *Brucella suis* [3] and experimentally by *Brucella abortus* [66]. The United States is free from the disease in domestic swine contrarily to feral swine which is a reservoir of the disease and represents an increasing risk of spill-over [67,68]. Considering the expanding number of the wild swine population as well as the potential for spill-over to domestic swine or to cattle [69,70], the need of protecting domestic swine especially outdoor domesticated swine is necessary. Serious economic loss will be faced if the domestic swine industry is affected by brucellosis [17].

Vaccination has demonstrated to be an efficacious way to control the disease and *Brucella* live attenuated vaccines have been broadly used in different species [62]. Although the *B. abortus* S19 vaccine played a key role reducing the disease in cattle [2], major drawbacks of inducing abortion in pregnant animals [55] and difficulties in discriminating vaccinated from infected animals via serological tests [2] limited the use of *B. abortus* S19 vaccine in the USA. However, worldwide S19 is still being used as an effective way to limit the burden of brucellosis [30]. Currently there is no commercially available vaccine to control swine brucellosis in the USA or elsewhere except the *B. suis* S2 vaccine which is only being used in China [36]. Thus, there is a critical need to develop a vaccine that will not only prevent the disease but also nullify major drawbacks like abortions, excretion of vaccine strains to the environment via body secretions like milk, urine and vaginal discharges but also vertical transmission to the offspring.

Type IV Secretion System (T4SS) are multiprotein complexes identified in several Gram-negative bacteria such as *Brucella spp*. and essential for their pathogenicity [73]. The Type IV Secretion System (T4SS) is encoded by the *virB* operon which is a key virulence factor [39]. The *jbR*, a LuxR family regulator, is known to regulate *virB* expression [74] and has an important role in the virulence and survival of *Brucella* in the mouse model [45]. Thus, a knock-out *vjbR* mutant vaccine (Δ*vjbR*) to attenuate organism virulence and reduce the possibility of inducing disease was developed by our laboratories that proved to confer a strong protective immune response against wild type challenge [48]. In order to enhance the efficacy of the Δ*vjbR* mutant vaccine, microencapsulation was established making it a potential option for a sustained and controlled vaccine delivery system [50]. Considering the promising results of the S19 Δ*vjbR* vaccine candidate in the mouse model [48], we investigated the potential use of the mutant vaccine under two different formulations, encapsulated and unencapsulated, in comparison with the *Brucella abortus* S19, in a natural host model to evaluate the safety of both vaccines.

As swine is the preferred host of *B. suis* [3], it is important to mention that the CDC and the USDA classifies *B. suis* as a select agent and it is prohibited to use this strain in BSL-2 facilities [75]. This major reason oriented the hypothesis of using *B. abortus* as another *Brucella* specie that can be used in BSL-2, but is also known to cause infection in swine [66]. Since *B. abortus* S19 is part of attenuated strains that is excluded from the Select Agent list and can be used under BSL-2 conditions not only experimentally but potentially during vaccine manufacturing [33,34], we sought to

evaluate the clinical safety of subcutaneous injection of live attenuated *B. abortus* S19 Δ*vjbR* at mid-gestation in pregnant gilts with a single dose of $1x10^{10}$ CFU per animal in comparison with *B. abortus* S19 commercial vaccine. In fact, during the early stage of the development of a live attenuated vaccine, one of the requirements is the demonstration of safety of any vaccine candidate [76].

Abortion is one of the major parameters to consider while evaluating the safety of a vaccine candidate for brucellosis. Abortion caused by natural *B. suis* infection in swine has been described at late gestation, if infection occurs after 35 days of pregnancy [15]. Vaccination with S19 has only been described in a study conducted in an endemic farm with brucellosis in 1948, during which abortions occurred while vaccinating pregnant sows with S19. Therefore, it is not clear whether abortion was due to vaccination with S19 or to a natural exposure. Consequently, we expected to see abortion in S19 vaccinated animals, as reported earlier in other species different than swine [55,77–80]. It is widely known that S19 induces abortion in pregnant cattle and bison during pregnancy [55,56,81]. In fact, abortion was reported while vaccinating adult pregnant cattle with S19 at a rate of 22% with 5.8×10^9 CFU/ animal [30,82]. Even a reduced dose of 3.0×10^8 organisms was able to induce abortions among pregnant cows vaccinated subcutaneously with S19 [79]. 58% of pregnant bison cows between 90-120 days of pregnancy were seen to abort starting from 60 days post-vaccination with 5.3×10^8 CFU of *Brucella abortus* S19 [55]. Additionally, abortion was seen following subcutaneous immunization of pregnant reindeer with 1.2×10^8 CFU of *Brucella abortus* S19 [77,83]. In the present study, vaccination of pregnant gilts with a high dose

of 1×10^{10} CFU/ animal of either S19 or with S19 Δ*vjbR* did not induce abortion among all animals contrarily to other species (Table 1). Interestingly, similar results of lack of abortion were described while vaccinating pregnant longhorn antelopes with the same dose of *Brucella abortus* S19 used in this study [80]. Behavioral change among all primiparous gilts used in this study was observed after farrowing causing traumatic post-partum deaths in piglets. This can be related to the susceptibility of primiparous to post-farrowing stress reflected by maternal infanticide of the gilt ranging from crushing or overlaying to aggressive biting of the offspring [84–86] and it is not considered to be a consequence of vaccination. Stillbirths evidenced by the lung test [60] were described among piglets of vaccinated gilts with S19 as well as the control group suggesting that this side effect is not related to vaccination. In fact, under normal conditions, stillborn piglets increase when they are born from primiparous gilts [84–86]. Since induction of abortion or adverse pregnancy outcomes were not observed in any of the vaccination groups, efficacy studies that include the potential use of S19 or S19Δ*vjbR* in swine is the next logical step towards the development of such a vaccine.

Undulant fever is one of the major symptoms of *Brucella* infection in humans [87]. Not only natural infection but also exposure of human to LAV *Brucella abortus* S19 is characterized by an undulant fever. In fact, 53% of vaccine-manufacturing laboratory employees in Argentina demonstrated an undulant fever as a major symptom after accidental exposure to *Brucella abortus* S19 vaccine [88]. However, fever has not been well investigated in animal brucellosis. In a recent study, an increase in body temperatures was observed in pregnant heifers within 1 to 2 days post-vaccination with

Brucella abortus S19 and it returned to normal right after that until calving [89]. Also, ewes vaccinated with *Brucella abortus* S19 demonstrated a rise of body temperature that reached 41.1ºC [90]. To the best of our knowledge, this is the first study investigating body temperature fluctuations and fever as a potential side effect related to vaccination in swine. In this study, we monitored daily body temperature of gilts and we did not detect fever in any animals throughout the duration of vaccination (Fig 1). Gilts body temperature was monitored via rectal temperature and the threshold of the maximum normal temperature was set at 38.8ºC [54]. It has been demonstrated by our study that vaccination with S19 or S19 Δ*vjbR* did not elicit any fever and body temperature remained normal and statistically insignificant while comparing them to the control group, suggesting that vaccination with S19 or S19Δ*vjbR* is not associated with fever in swine. Whether it is a predictor of abortion or not, is yet to be elucidated since none of the animals aborted in this study. Although a decrease of body temperature within the normal range was observed during 2 and 3 week post vaccination below, we were able to associate it with a decrease in environmental temperature during the study since animals were housed outdoors.

Brucellosis is characterized by excreting the bacteria to the environment through secretions for instance milk, urine, or vaginal discharges [91,92]. Shedding of vaccine strains in those secretions is one of the most important parameters to consider while developing a vaccine, since contamination of the environment including water resources and pastures could potentially pose a risk to other non-target species residing in the same premises including wildlife and humans. In our study, the investigation of the potential

excretion of the vaccine strains in vaginal swabs was assessed. Vaginal swabs were chosen because animals were pregnant and it has been shown that reproductive tissues are often heavily infected with *Brucella* spp and can be excreted in vaginal mucus [93–95]. For instance, a previous report described shedding of *Brucella abortus* S19 vaccine strain in vaginal mucus of heifers [96]. Also, *Brucella abortus* S19 was isolated from a vaginal swab specimen after 51 days from vaccination of a reindeer with the same vaccine strain [77]. In swine, vaginal shedding was only described in an animal inoculated experimentally with *Brucella abortus* wild type via the subcutaneous route [66]. In this study, the assessment of vaginal secretions of S19 and S19 Δ*vjbR* was established during early weeks of vaccination until 6 week post-vaccination. None of the vaccinated animals shed any vaccine strains in the vagina at any time point to detectable levels.

Brucellosis is accompanied by colonization of major tissues [97,98] and considered as an occupational disease that can affect workers such as butchers in close contact with animal organs. Thus, we wanted to investigate if the vaccine strains were still circulating in different organs after delivery which can represent a potential risk to humans while handling carcasses of vaccinated animals. At 7 week post-vaccination, the vaccine strains were not isolated from any of the major tissues of gilts suggesting that both S19 and S19Δ*vjbR* strains are not present or below the limit of detection in gilts by 7 week post vaccination and that S19, S19Δ*vjbR* encapsulated and S19Δ*vjbR* unencapsulated vaccine candidate are safe for swine carcass handlers.

Major expected lesions that can affect animals either with *Brucella* wild type or S19 are placentitis, endometritis, granulomatous hepatitis and splenomegaly [48,99]. We corroborated the clearance results with unremarkable histologic findings of major tissues of gilts.

Aborted fetuses as well as infected offspring are not only hazardous to humans but also to the environment. This serious issue needs to be considered while vaccinating animals with LAV. Vaccination of pregnant cattle with *Brucella abortus* S19 results in vertical transmission to fetuses and the S19 vaccine strain can be commonly isolated from gastric contents of aborted products [55]. In swine, transplacental transmission was described in naturally infected sows [12,100] and *Brucella Suis* S2, the only LAV available against swine brucellosis in China, is known to induce abortion while administered SQ or IM [36]. For that, we cultured homogenate tissues of piglets within the first hours of birth and did not isolate any *Brucella* for the most suspected tissues that can be colonized namely the stomach and gastric contents and the umbilicus or lungs [18]. Unmarked histologic lesions of the same tissue sections of piglets corroborated that result and therefore, we can suggest that S19 and S19 Δ*vjbR* did not cross the placental barrier while being inoculated at midgestation in pregnant swine.

The immune response triggered by *Brucella* infection and vaccination was broadly studied in the mouse model [101]. It was established that the principal role to overcome the infection is played by cellular immunity and secondary the humoral response [102]. Experimental infection of swine with 2×10^7 CFU of *Brucella suis* biovar 2 through the conjunctival route demonstrated a long lasting humoral response of 21

weeks post-inoculation by Rose Bengal Test (RBT) and iELISA [103]. Immunization with smooth LAV elicits a humoral response toward the O-polysaccharide of the LPS [102,104]. In order to monitor the kinetics of humoral response elicited by vaccination in swine and to see if there was any correlation of immune protection that could predict efficacy in future studies, we studied anti-*Brucella* antibody responses triggered by vaccination using two common validated serological tests in cattle and small ruminants [105]. The Rose Bengal Test (RBT) which was used in Europe to screen for swine brucellosis [106] demonstrated that all gilts involved in this study have not previously been exposed to *Brucella* antigens eliciting a negative agglutination response at pre-vaccination. At 2 week post-vaccination, the agglutination response was the strongest and started to decrease starting from 4 week and was negative at 6 week post-vaccination. The post-vaccinal humoral reaction in this study using S19 and S19Δ*vjbR* vaccine candidates seems to be transient while comparing it to experimental infection in swine with *Brucella abortus* which demonstrated an agglutination response that lasted more than 10 week with RBT [66]. These findings correlate with the unpublished results on swine vaccinated with S19 mentioning that the agglutinin response dropped rapidly after 4 weeks of vaccination [32]. In cattle, subcutaneous vaccination with $3x10^9$ CFU of strain S19 bacteria elicited an intense agglutination with RBT at 4 weeks post-vaccination (30 days) in 25 animals and started to decrease starting from 12 weeks post-vaccination until it became negative at 38 weeks post-vaccination [107]. This demonstrates that vaccination with *Brucella abortus* S19 in cattle elicits a prolonged humoral response as opposed to swine. The iELISA which is a more sensitive test based

upon the reactivity toward the LPS of *Brucella abortus* 2308 lysate was used to confirm the vaccinal status of the animals but also study the kinetics of both anti-*Brucella* IgM and IgG. Our findings revealed not only that the animals were not previously into contact with *Brucella* antigens but also they had a peak at 2 weeks post-vaccination same as the RBT. Starting from 4 weeks, the anti-*Brucella* IgM demonstrated similar kinetics to the RBT characterized by a decrease in OD values among all the treatment groups and reached negativity at 6 week post vaccination. On the other hand, the IgG OD values peaked at 2 week post-vaccination with the vaccine candidates as well as S19 and started to decrease starting from 4 weeks but did not reach negativity by 6 weeks. In comparing species vaccinated with S19, cattle demonstrated a strong total IgG response at 28 days post-vaccination that decreased significantly after 30 weeks post-vaccination [31]. In regards to the discrepancy of the results between the RBT and the anti-*Brucella* IgG iELISA, we studied the correlation between the two tests and we found out that there's a substantial correlation during the 2 week and 4 week post-vaccination interval and a poor correlation at 6 week post-vaccination. Since the vaccination elicited a transient humoral response, we can easily distinguish between vaccinated animals and infected ones. Infected animals with *Brucella* will have a sustained humoral response as opposed to vaccinated animals with S19 or S19 Δ*vjbR* using the iELISA [103]. Practically, a screening test within 2 and 4 week post-vaccination interval can be introduced in the field and can serve as a DIVA strategy either while vaccinating swine with S19 or S19 Δ*vjbR*.

One major drawback can be encountered while using the iELISA or the RBT is the inability to differentiate *Brucella* infection or vaccination with S-LPS LAV from *Escherichia coli* O:157, some strains of *Escherichia hermanni* and *Salmonella* group N (O:30), *Stenotrophomonas maltophila*, and *Vibrio cholerae* O1 and especially *Yersinia enterocolitica* O:9 infection in swine [63,106,108]. To address this issue, we investigated the potential use of purified *vjbR* protein as an immunogenic marker to differentiate vaccinated animals from infected ones by developing a serological test that would allow this based on studies on humans that proved that the *vjbR* is highly immunogenic and reactive toward sera of infected humans with *Brucella* [52]. Hence, we expressed the *vjbR* in *E. coli* and in yeast. Purified *vjbR* from yeast was used to produce rabbit anti-*vjbR* polyclonal and as a potential source of protein because we faced non-specific reactivity with *vjbR* expressed in *E. coli*. To characterize the *vjbR* protein in *E. coli*, liquid chromatography–mass spectrometry (LCMS) was employed to confirm the use of the purified *vjbR* for the DIVA serological tests. Our findings asserted with high confidentiality that we purified the correct protein (*VjbR* was identified by 19 unique spectra and 9 unique peptides and the peptide coverage was 38%). Additionally, anti-histag western blot and rabbit anti-*vjbR* toward the *vjbR* demonstrated the same result. The *vjbR* recombinant protein was then used in a western blot with pre-vaccinated sera of all gilts and demonstrated non-specific binding to the *vjbR* protein with a variation of the intensity of the bands observed, despite multiple titrations of both primary and secondary antibodies. Interestingly one animal of the control group did not react with the *vjbR* protein during pre-vaccination and 2 week post-inoculation. Post-vaccination

sera of the second week of all gilts were also analyzed via western blot for the reactivity toward the *vjbR* protein expressed in *E. coli* and demonstrated comparable results to the pre-vaccination.

These findings oppose a previous study on the mouse model considering the usefulness of the *vjbR* protein as a diagnostic antigen to differentiate vaccinated mice with 16M Δ*vjbR* from ones [109]. Closely related genera of bacteria can be misidentified as *Brucella* [110,111]. Therefore, we correlated those results to the potential previous contact of the gilts used in this study to *Brucella*-like bacteria sharing identical sequence of the *vjbR* protein. We identified 3 potential bacteria with BLASTp: *Ochrobactrum anthropi* was selected because it is closely related and sometimes misidentified to *Brucella* but also it is found in the soil which increases the chances of contact of swine with the bacteria [110,111]. *Pseudomonas aeruginosa* was selected because it gives false negative response in low specificity serodiagnosis assays considering the fact that it shares the outer membrane vesicles (OMVs) with *Brucella* [112] and *E. coli* K-12 because we suspected a residual effect from expressing the *vjbR* protein in *E. coli* and that is why we expressed the *vjbR* protein in yeast as well. Sera from S19 and S19 Δ*vjbR* encapsulated gilts that demonstrated intense cross-reactivity with the *vjbR* protein at the pre-vaccination were exposed to these 3 bacterial lysates. No reactivity toward *Ochrobactrum anthropi*, *Pseudomonas aeruginosa* and *E. coli* K-12 was noticed. As it was shown in the case of BP26, an immunogenic protein of *B. melitensis*, sera from *Brucella*-free sheep and *B. melitensis* infected sheep cross-reacted with the protein. A substitution of amino acids from the entire BP26 while keeping the region of BP26

between amino acids 55 and 152 allowed to avoid the false positive reaction of *Brucella*-free sheep sera [113].

Further investigations using the *vjbR* protein for epitope mapping [114] will be undertaken to develop a reliable serological diagnostic tool that distinguishes infected animals from vaccinated ones and therefore assesses the DIVA capability of the S19 Δ*vjbR* vaccine candidate in natural hosts.

Overall, the vaccine candidates proved to be safe and did not induce any undesirable side effect. Vaccination with S19Δ*vjbR* vaccine candidates triggered a transient anti-*Brucella* humoral response comparable to the one elicited by S19 in swine. For that, an efficacy study will be undertaken to evaluate the protective aspect of the vaccine candidate under the two formulations. The variable cross-reactivity observed in pre-vaccinated sera with the *vjbR* protein compromised the DIVA ability of the vaccine candidates in this study. Therefore, an epitope mapping is essential to identify the protein binding site that causes the non-specific reactivity and substitute it to develop a DIVA serological tool using the S19 Δ*vjbR* vaccine candidate.

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APPENDIX A

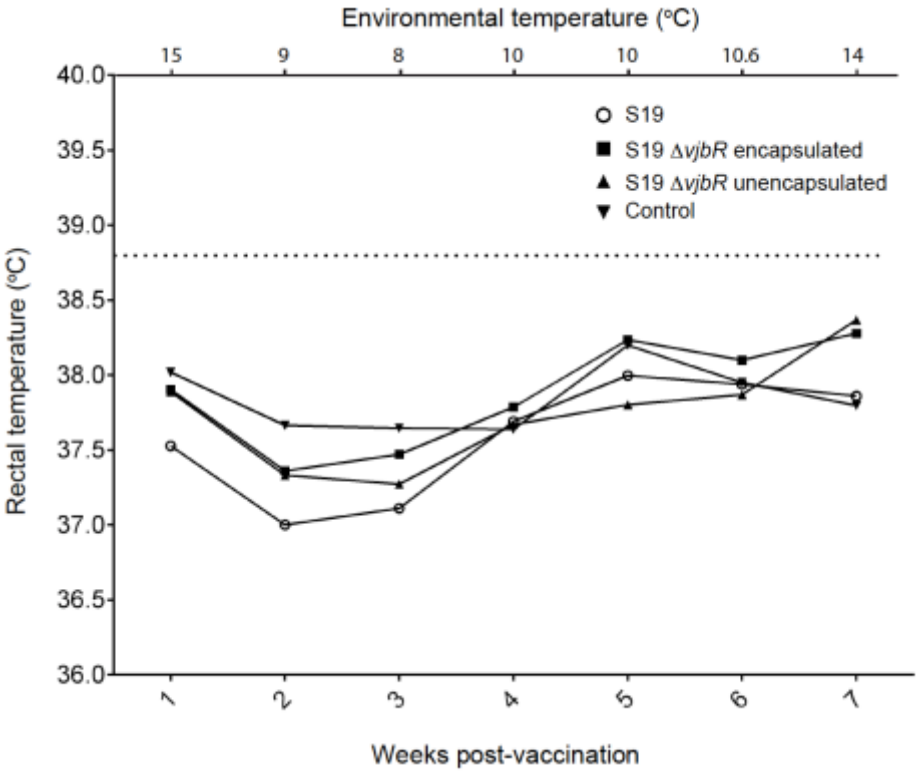

Fig. 1. Average rectal temperature in gilts vaccinated with live attenuated vaccine formulations. Results are expressed as mean of weekly body temperatures of each group. Temperature above 38.8 ºC is considered fever. Statistical analysis was performed using the one-way analysis of variance (ANOVA) test and Dunn's multiple comparisons. No significant difference was observed between groups

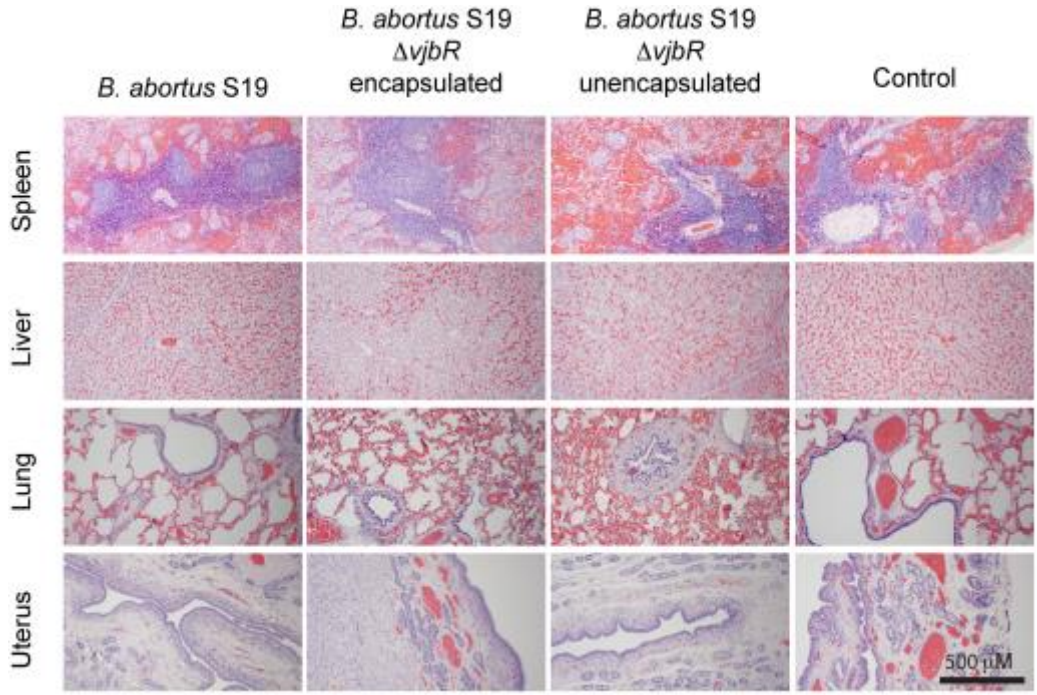

Fig. 2. Histological analysis of spleen, liver, lung and uterus from gilts inoculated with 1) S19, 2) S19 Δ*vjbR* encapsulated, 3) S19 Δ*vjbR* unencapsulated and 4) Empty capsule (control) at 5 days post-delivery. None of the gilts inoculated with the different vaccine strains had any significant histopathological changes in the major organs.

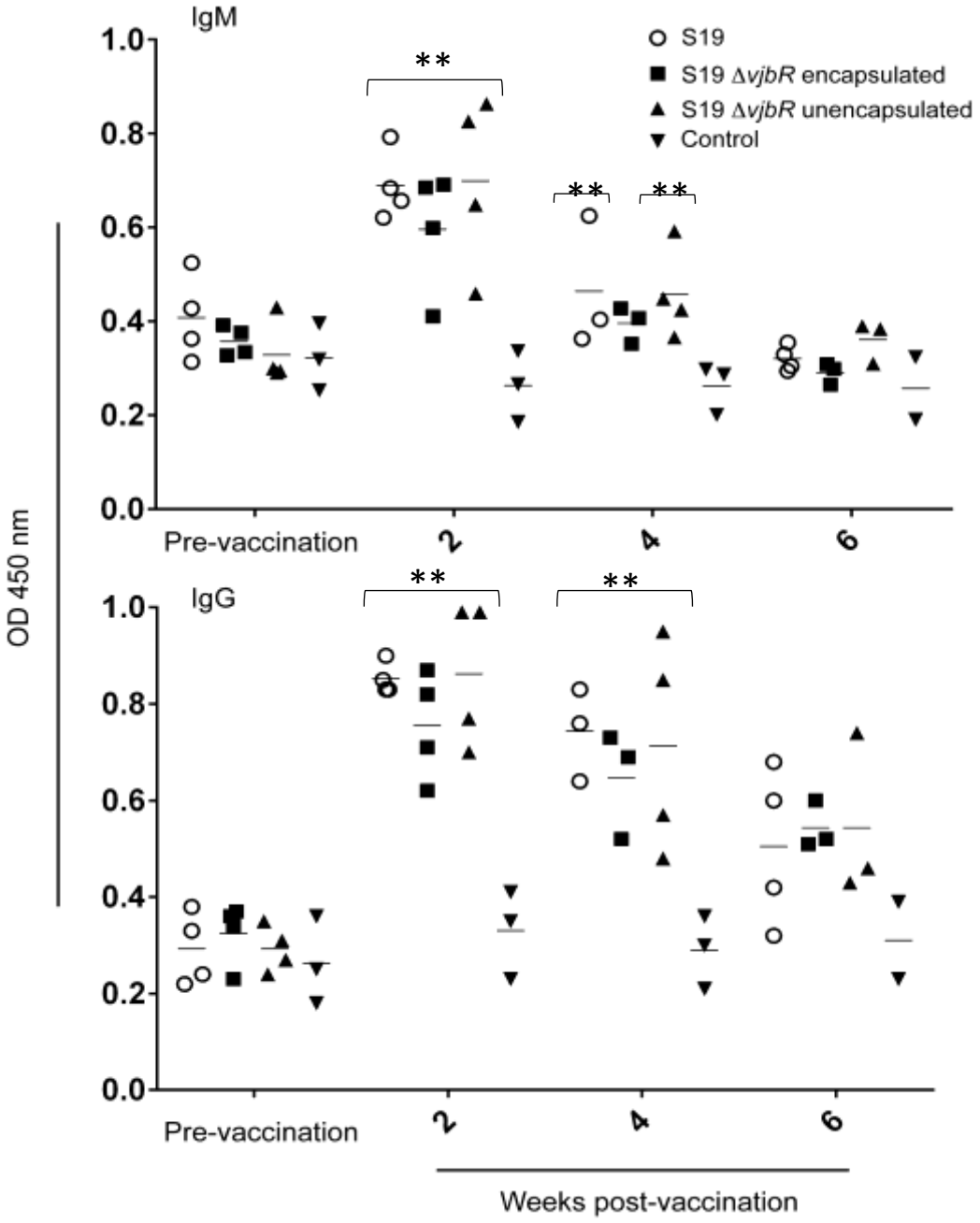

Fig. 3. Anti-*Brucella* specific IgM and IgG responses in serum samples from individual gilts immunized with different *B. abortus* vaccinates and individual gilts from control group. Results are expressed as mean of individual sera OD values. Statistical analysis was done by comparing the mean of all groups between each other at different time points by using the two-way analysis of variance (ANOVA) test and Tukey's multiple

comparisons. **Values are statistically different (P<0.01) from the control at different time points.

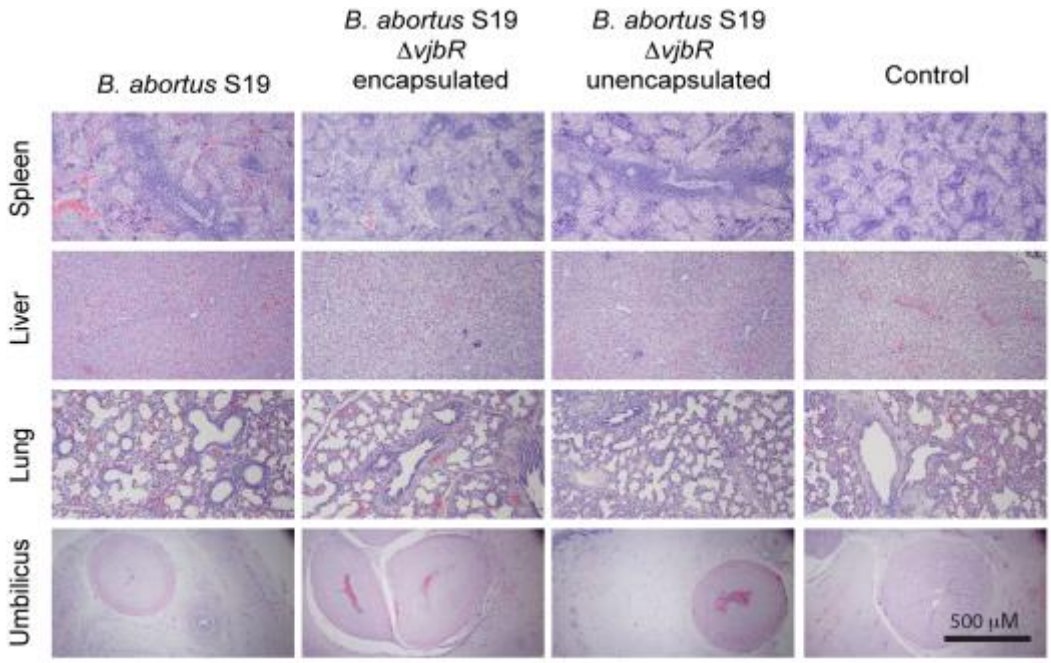

Fig. 4. Histological analysis of spleens, livers, lungs (10x) and umbilical cords (4x) from piglets of gilts inoculated with 1) S19, 2) S19 Δ*vjbR* encapsulated, 3) S19 Δ*vjbR* unencapsulated and 4) Control.

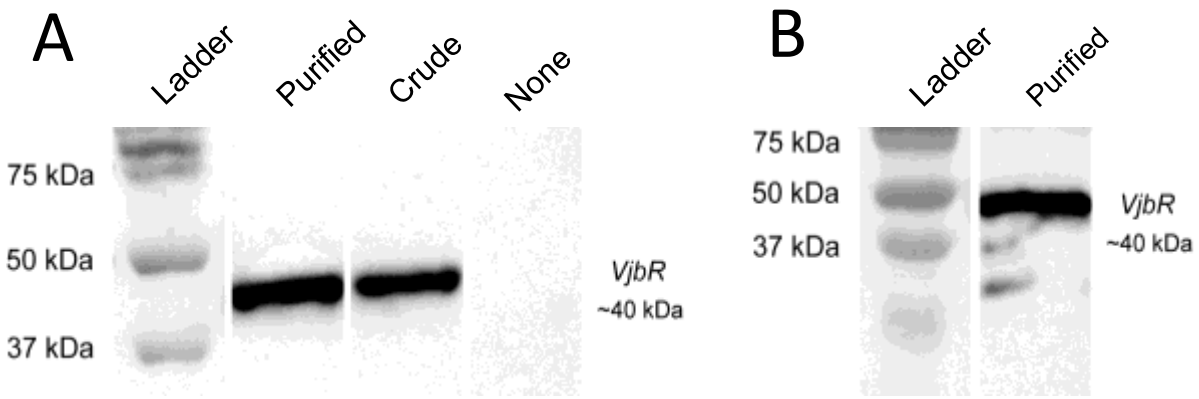

Fig. 5. Immunodetection of the *vjbR* protein by Western blot analyses. (A) Anti-histag specific Western blot. (B) *vjbR* purified protein reactivity with rabbit anti-*vjbR* polyclonal antibody. The purified *vjbR* protein was expressed in *E. coli*. The reacting *vjbR* protein size is estimated at ～40 kDa

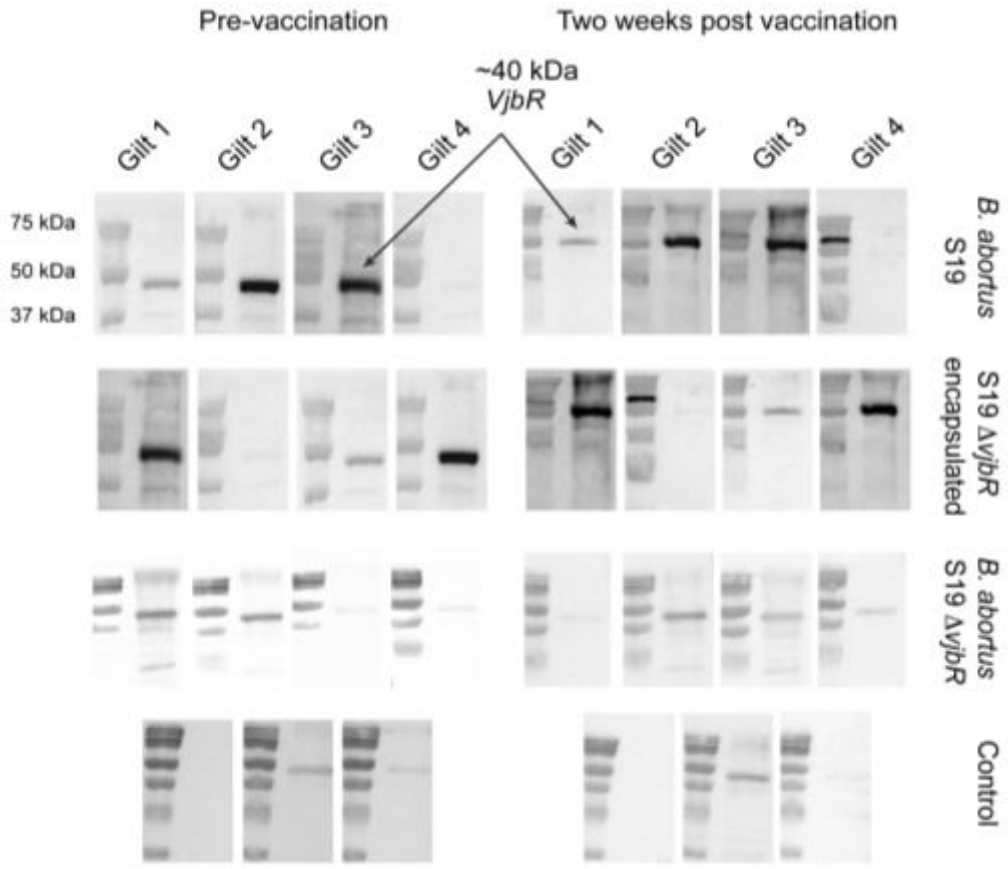

Fig. 6. Immunoreactivity of pre-vaccinated and 2nd week post-vaccinated gilt sera with S19, S19 Δ*vjbR* encapsulated or unencapsulated and empty capsule to the *vjbR* protein. A dark band is showing at the *vjbR* protein size (~40 kDa) at pre-vaccination with variable intensity.

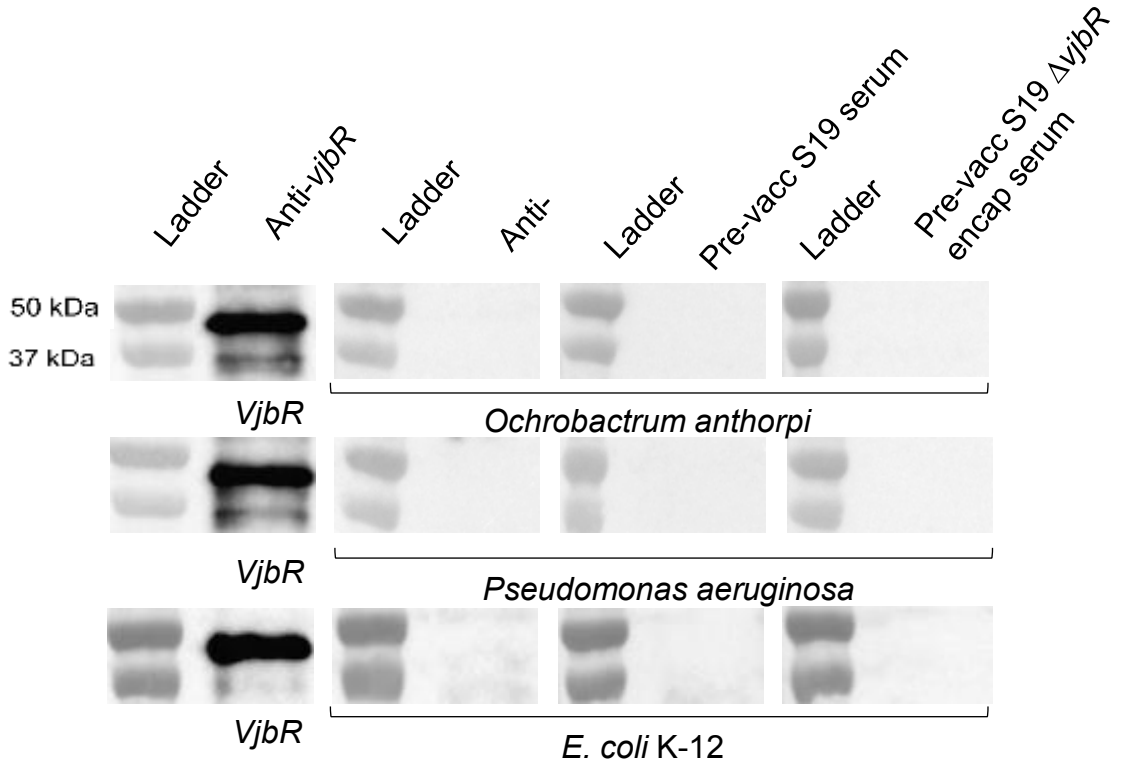

Fig. 7. Reactivity of rabbit anti-*vjbR*, pre-vaccinated sera of S19 and S19 Δ*vjbR* gilts with *Ochrobactrum anthorpi*, *Pseudomonas aeruginosa* and *E. coli* K-12. *VjbR* purified protein exposed to rabbit anti-*vjbR* was used as a positive control.

APPENDIX B

Table 1
Litter size and number of births from pregnant gilts after farrowing.

Group	Animal Number	# of piglets per gilt	Total litter size	Litter size (Mean ± SD)	# of abortions	# of post-partum deaths	# of stillbirths
S19	1	13	49	12.25 ± 2.5	0/49	3	1/6 (16.66%)
	2	12				1	
	3	9				2	
	4	15				0	
S19 Δ*vjbR* encapsulated	1	13	49	12.25 ± 0.96	0/49	1	0/3 (0%)
	2	12				2	
	3	11				0	
	4	13				0	
S19 Δ*vjbR* unencapsultated	1	12	45	11.25 ± 2.99	0/45	1	0/5 (0%)
	2	8				1	
	3	15				1	
	4	10				2	
Control	1	11	38	12.7± 3.79	0/38	10	2/12 (16.66%)
	2	10				0	
	3	17				2	

p-value of 0.9
Abortion is defined by expulsion of dead fetuses prior to normal delivery (normal delivery is estimated to occur at 115 days of pregnancy in swine).
Post-partum deaths are defined by the normal delivery of either hypoxic (non-breathing) piglets classified as stillbirths or traumatic deaths by crushing.

Table 2
Vaccine shedding in pregnant gilts through vaginal secretions after vaccination.

Group	Animal Number	Pre-vaccination	2 week post vaccination	4 week post vaccination	6 Week post vaccination
S19	1	-	-	-	-
	2	-	-	-	-
	3	-	-	-	-
	4	-	-	-	-
S19 Δ*vjbR* encapsulated	1	-	-	-	-
	2	-	-	-	-
	3	-	-	-	-
	4	-	-	-	-
S19 Δ*vjbR* unencapsultated	1	-	-	-	-
	2	-	-	-	-
	3	-	-	-	-
	4	-	-	-	-
Control	1	-	-	-	-
	2	-	-	-	-
	3	-	-	-	-

Table 3
Bacterial colonization in multiple tissues from gilts immediately after delivery (7 week post-vaccination).

Group	Blood	Liver	Lung	Spleen	Mammary LN	Inguinal LN	Uterus	Placenta
S19	-	-	-	-	-	-	-	-
S19 Δ*vjbR* encapsulated	-	-	-	-	-	-	-	-
S19 Δ*vjbR* unencapsultated	-	-	-	-	-	-	-	-
Control (Empty capsule)	-	-	-	-	-	-	-	-

Table 4
Rose Bengal screening in vaccinated gilts at 0, 2, 4 and 6 week post-vaccination.

Group	Animal Number	Pre-vaccination	2 week post vaccination	4 week post vaccination	6 Week post vaccination
S19	1	-	+++	++	-
	2	-	+++	n/a	-
	3	-	+++	+	-
	4	-	+++	++	-
S19 Δ*vjbR* encapsulated	1	-	+++	+	n/a
	2	-	++	++	-
	3	-	++	-	-
	4	-	++	n/a	-
S19 Δ*vjbR* unencapsultated	1	-	+++	+	-
	2	-	++++	+++	n/a
	3	-	++++	+++	-
	4	-	-	-	-
Control	1	-	-	-	-
	2	-	-	-	n/a
	3	-	-	-	-

Samples from animals represented by n/a indicate gilts that the collection of blood was not available

++++/+++ strong agglutination
++ mild agglutination
+ weak agglutination
- no agglutination

Table 5
Comparison of the iELISA and RBT using Bayesian analysis for vaccination screening.

Estimates	Pre-vaccination	2 Week Post-vaccination	4 Week Post-vaccination	6 Week Post-vaccination
Kappa 'κ'	1	0.8	0.65	0
Agreement	Perfect	Substantial	Substantial	Poor

Table 6
Vertical colonization of piglet major tissues immediately after delivery.

Group	Total litter size	Liver	Lung	Spleen	Umbilicus	Kidney	Gastric contents
S19	49	0/49	0/49	0/49	0/49	0/49	0/49
S19 Δ*vjbR* encapsulated	49	0/49	0/49	0/49	0/49	0/49	0/49
S19 Δ*vjbR* unencapsultated	45	0/45	0/45	0/45	0/45	0/45	0/45
Control (Empty capsule)	38	0/38	0/38	0/38	0/38	0/38	0/38